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REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

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International Application No. **PCT/US 98"/16181**

International Filing Date

05.08.98
05 AUG 1998

**PCT INTERNATIONAL
APPLICATION RO/US**

Name of receiving Office and "Patent Cooperation Treaty" Application n°

Applicant's or agent's file reference
(if desired) (12 characters maximum) 09143/005WO1

Box No. I TITLE OF INVENTION
DIETARY SUPPLEMENTS CONTAINING NATURAL INGREDIENTS

Box No. II APPLICANT

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

Melaleuca, Incorporated
3910 S. Yellowstone Hwy.
Idaho Falls, ID 83402-6003
US

☐ This person is also inventor.

Telephone No.

Facsimile No.

Teleprinter No.

State (that is, country) of nationality:
US

State (that is, country) of residence:
US

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)

PERKES, Lynn
308 West 1st North
Rexburg, ID 83440
US

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only (If this check-box is marked, do not fill in below.)

State (that is, country) of nationality:
US

State (that is, country) of residence:
US

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☐ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as:

☒ agent ☐ common representative

Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

ANDERSON, Richard J.
Fish & Richardson P.C., P.A.
60 South 6th Street, Suit 3300
Minneapolis, MN 55402
US

Telephone No.
612/335-5070

Facsimile No.
612/288-9696

Teleprinter No.

☐ Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No.V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

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- ☒ **AP ARIPO Patent:** GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
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- ☒ **OA OAPI Patent:** BF Burkina Faso, BJ Benin, CF Central African Republic, CG Congo, CI Côte d'Ivoire, CM Cameroon, GA Gabon, GN Guinea, ML Mali, MR Mauritania, NE Niger, SN Senegal, TD Chad, TG Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT (if other kind of protection or treatment desired, specify on dotted line)

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- | | |
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- ☐

Precautionary Designation Statement: In addition to the designations made above, the applicant also makes under Rule 4.9(b) all other designations which would be permitted under the PCT except any designation(s) indicated in the Supplemental Box as being excluded from the scope of this statement. The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. (Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)

Supplemental Box *If the Supplemental Box is not used, this sheet need not be included in the request.*

1. If, in any of the Boxes, the space is insufficient to furnish all the information: in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient, in particular:

- (i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available: in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below;
- (ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is applicant;
- (iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America: in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) name, the State(s) (and/or, where applicable, ARIPO, Eurasian, European or OAPI patent) for the purposes of which the named person is inventor;
- (iv) if, in addition to the agent(s) indicated in Box IV, there are further agents: in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;
- (v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V., the name of the United States of America is accompanied by an indication "continuation" or "continuation-in-part": in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;
- (vi) if, in Box No. VI, there are more than three earlier applications whose priority is claimed: in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI;
- (vii) if, in Box No. VI, the earlier application is an ARIPO application: in such case, write "Continuation of Box No. VI", specify the number of the item corresponding to that earlier application and indicate at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed.

2. If, with regard to the precautionary designation statement contained in Box No. V, the applicant wishes to exclude any State(s) from the scope of that statement: in such case, write "Designation(s) excluded from precautionary designation statement" and indicate the name or two-letter code of each State so excluded.

3. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty: in such case, write "Statement concerning non-prejudicial disclosures or exceptions to lack of novelty" and furnish that statement below.

This is a Continuation-In-Part of U.S. Application Serial Number 08/907,317, filed 6 August 1997.



B x No. VI PRIORITY CLAIM		<input type="checkbox"/> Further priority claims are indicated in the Supplemental Box.		
Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country	regional application: regional Office	international application: receiving Office
item (1) (06.08.97) 06 August 1997	08/907,317	US		
item (2)				
item (3)				

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office) identified above as item(s): item (1)

* Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY	
Choice of International Searching Authority (ISA) (if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used): ISA/US	Request to use results of earlier search; reference to that search (if an earlier search has been carried out by or requested from the International Searching Authority): Date (day/month/year) Number Country (or regional Office)

Box No. VIII CHECK LIST: LANGUAGE OF FILING	
This international application contains the following number of sheets: request : 4 description (excluding sequence listing part) : 30 claims : 5 abstract : 1 drawings : 8 sequence listing part of description : _____ Total number of sheets : 48	This international application is accompanied by the item(s) marked below: 1. <input checked="" type="checkbox"/> fee calculation sheet 2. <input type="checkbox"/> separate signed power of attorney 3. <input type="checkbox"/> copy of general power of attorney; reference number, if any: 4. <input type="checkbox"/> statement explaining lack of signature 5. <input type="checkbox"/> priority document(s) identified in Box No. VI as item(s): 6. <input type="checkbox"/> translation of international application into (language): 7. <input type="checkbox"/> separate indications concerning deposited microorganism or other biological material 8. <input type="checkbox"/> nucleotide and/or amino acid sequence listing in computer readable form 9. <input checked="" type="checkbox"/> other (specify): Transmittal Letter
Figure of the drawings which should accompany the abstract:	Language of filing of the international application: US

Box No. IX SIGNATURE OF APPLICANT OR AGENT	
Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).	
Melaleuca, Incorporated  Richard J. Anderson Attorney for Applicant	PERKES, Lynn  Richard J. Anderson Attorney for Applicant

1. Date of actual receipt of the purported international application: 45 Rec'd PCT/PTC 05 AUG 1998	2. Drawings: <input type="checkbox"/> received: <input type="checkbox"/> not received:
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:	
4. Date of timely receipt of the required corrections under PCT Article 11(2):	
5. International Searching Authority (if two or more are competent): ISA/US	6. <input type="checkbox"/> Transmittal of search copy delayed until search fee is paid

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Form PCT/RO/101 (last sheet) (July 1998) LegalStar 1998, Form PCTREQ See Notes to the request form

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(for several international applications filed under the Patent Cooperation Treaty)

(PCT Rule 90.5)

The undersigned person(s):

(Family name followed by given name; for a full legal entity, full official designation. The address must include postal code and name of country.)

Melaleuca, Incorporated
3910 S. Yellowstone Hwy.
Idaho Falls, ID 83402-6003
US

hereby appoint(s) the following person as:

☒ agent

☐ common representative

Name and address

(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

Richard J. Anderson, Mark S. Ellinger, Ronald C. Lundquist,
Steven J. Shumaker, Dorothy P. Whalen, Monica McCormick Graham and
Ruffin B. Cordell
FISH & RICHARDSON P.C., P.A.
60 South Sixth Street, Suite 3300
Minneapolis, MN 55402
US

to represent the undersigned before

☒ all the competent International Authorities

☐ the International Searching Authority only

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in connection with any and all international applications filed by the undersigned with the following Office

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Signature(s) (where there are several persons, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading this power):


By: Kenneth J. Sheppard
Title: U.P. and General Counsel

Date:

Oct. 12, 1998

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PCT/US 98/16181
RO/US 26 OCT 1998

GENERAL POWER OF ATTORNEY

(for several international applications filed under the Patent Cooperation Treaty)

(PCT Rule 90.5)

The undersigned person(s):

(Family name followed by given name; for a full legal entity, full official designation. The address must include postal code and name of country.)

Lynn Perkes
308 West 1st North
Rexburg, ID 83440
US

hereby appoint(s) the following person as:



agent



common representative

Name and address

(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)

Richard J. Anderson, Mark S. Ellinger, Ronald C. Lundquist,
Steven J. Shumaker, Dorothy P. Whalen, Monica McCormick Graham and
Ruffin B. Cordell
FISH & RICHARDSON P.C., P.A.
60 South Sixth Street, Suite 3300
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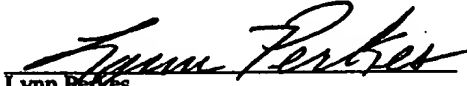
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Signature(s) *(where there are several persons, each of them must sign; next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading this power):*


Lynn Perkes

Date:

Oct. 8, 1998

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FEE CALCULATION SHEET

Annex t the Request

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International application No.

PCT/US 98"/1618

Date stamp of the receiving Office

05 AUG 1998

Applicant's or agent's
file reference

09143/005WO1

Applicant

M laleuca, Inc. and Lynn Perkes

CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE	240.00	<input type="checkbox"/> T	
2. SEARCH FEE	450.00	<input type="checkbox"/> S	
International search to be carried out by <u>US</u>			
<i>(If two or more International Searching Authorities are competent in relation to the international application, indicate the name of the Authority which is chosen to carry out the international search.)</i>			
3. INTERNATIONAL FEE			
Basic Fee			
The international application contains <u>48</u> sheets.			
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<u>18</u> x <u>\$10.00</u>	180.00	<input type="checkbox"/> b ₂	
remaining sheets additional amount			
Add amounts entered at b ₁ and b ₂ and enter total at B	635.00	<input type="checkbox"/> B	
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The international application contains <u>73</u> designations.			
<u>11</u> x <u>105.00</u>	1,155.00	<input type="checkbox"/> D	
number of designation fees payable (maximum 11)	amount of designation fee		
Add amounts entered at B and D and enter total at I	1,790.00	<input type="checkbox"/> I	
<i>(Applicants from certain States are entitled to a reduction of 75% of the international fee. Where the applicant is (or all applicants are) so entitled, the total to be entered at I is 25% of the sum of the amounts entered at B and</i>			
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5. TOTAL FEES PAYABLE	2,495.00		
Add amounts entered at T, S, I and P, and enter total in the TOTAL box	TOTAL		

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 35/78, 31/685, 31/715	A1	(11) International Publication Number: WO 99/07400 (43) International Publication Date: 18 February 1999 (18.02.99)
(21) International Application Number: PCT/US98/16181 (22) International Filing Date: 5 August 1998 (05.08.98) (30) Priority Data: 08/907,317 6 August 1997 (06.08.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/907,317 (CIP) Filed on 6 August 1997 (06.08.97) (71) Applicant (for all designated States except US): MELALEUCA, INCORPORATED [US/US]; 3910 S. Yellowstone Highway, Idaho Falls, ID 83402-6003 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): PERKES, Lynn [US/US]; 308 West 1st North, Rexburg, ID 83440 (US). (74) Agent: ANDERSON, Richard, J.; Fish & Richardson P.C., P.A., Suite 3300, 60 South 6th Street, Minneapolis, MN 55402 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: DIETARY SUPPLEMENTS CONTAINING NATURAL INGREDIENTS (57) Abstract The invention provides a dietary supplement comprising at least one flavonoid source and an enzyme, that is effective for inhibiting <i>in vivo</i> platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 30 mg/kg or less. The supplement may contain flavonoid sources found in grape seed extracts, grape skin extracts, bilberry extracts, ginkgo biloba extracts or the flavonoid quercetin. The supplement may also contain fungal proteases, acid stable proteases and bromelain. The invention further provides a method for using the dietary supplement and an article of manufacture containing the supplement.		

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- 1 -

**DIETARY SUPPLEMENTS CONTAINING NATURAL INGREDIENTS
BACKGROUND**

1. Technical Field

5 The invention relates to dietary supplements containing natural ingredients.

2. Background Information

Coronary artery disease, myocardial infarction, stroke, and other vascular occlusions are major health
10 concerns. A common characteristic of these diseases is the atherosclerotic process, or the narrowing of arteries. Blood platelets contribute to the development and progression of the atherosclerotic process by releasing growth factors, chemotactic substances and
15 other factors that accelerate the atherosclerotic process. In addition, platelet aggregation at or near the point of arterial damage contributes to the development of atherosclerosis and acute platelet thrombus formation.

20 Low density lipoprotein (LDL) cholesterol is also associated with atherosclerosis. It has been proposed that nonatherogenic LDL cholesterol circulating in the blood is converted to atherogenic LDL cholesterol through oxidation of polyunsaturated lipids, which leads to
25 modification of the apoprotein.

Physicians use various drugs, such as aspirin, to treat atherosclerotic conditions. Aspirin, however, is not without negative side effects including gastrointestinal irritation. Interventions such as
30 angioplasty are also available to dilate stenosed arteries thereby increasing blood flow. Interventional techniques, however, produce intimal and medial artery damage and expose thrombogenic surfaces. As such,

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restenosis and the incidence of sudden coronary death following angioplasty is a major concern for patients with known or suspected coronary artery disease.

Given the grave consequences of atherosclerosis and the costs associated with medical treatments, there is a need for pharmacologic and nutritional interventions that are useful for preventing the occurrence and reoccurrence of these conditions.

Epidemiological studies have noted an inverse correlation between the intake of dietary flavonoids from fruits and vegetables and death from coronary artery disease. This correlation is thought to arise from the antioxidant and platelet inhibition properties of flavonoids found in fruits and vegetables.

Certain flavonoids, including those found in grape seed and grape skin extracts, have been associated with the beneficial health effects observed for aspirin, but without the negative side effects attributed to aspirin. Nevertheless, flavonoid bioavailability or activity is low in many sources of flavonoids. As such, certain dietary sources of flavonoids require large doses to be useful. As a result, many sources of flavonoids are impractical, too costly, or both to be useful on a daily basis.

SUMMARY

The present invention involves the discovery that the combination of certain flavonoids and enzymes in the form of a dietary supplement reduces the dosage of supplement needed to effectively reduce platelet activity and LDL cholesterol oxidation in a mammal. The present invention further involves a method to treat conditions associated with platelet activity and LDL cholesterol oxidation by administering combinations of flavonoids and

- 3 -

enzymes to reduce platelet activity and LDL cholesterol oxidation.

In one aspect, the invention features a dietary supplement containing at least one flavonoid source and
5 an enzyme wherein the supplement is effective for inhibiting platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 30 mg/Kg or less. One example of a dietary supplement in accordance with the invention is PROVEXCV™. It is to be understood
10 that a dosage of supplement as used herein refers to the combined weight of the flavonoid source or sources and enzyme. Further, other ingredients such as fillers, lubricants, carriers and the like may be included as additional ingredients.

15 The flavonoid source or sources in the dietary supplement may be derived from grape seed extracts, grape skin extracts, ginkgo biloba extracts, bilberry extracts or quercetin. The enzymes may include fungal proteases, acid stable proteases, neutral stable proteases, alkaline
20 stable proteases or bromelain.

In another aspect, the invention features a dietary supplement containing at least one flavonoid source wherein the supplement is effective for inhibiting platelet activity and LDL cholesterol oxidation in a
25 mammal at a dosage of about 30 mg/Kg or less. Preferably, a dietary supplement in accordance with this aspect further includes an enzyme that substantially reduces the dosage required to achieve such inhibition.

In another aspect, the invention features a method
30 to inhibit platelet activity or LDL cholesterol oxidation or both in a mammal by administering a dietary supplement containing a flavonoid source and an enzyme wherein the supplement is effective for reducing platelet activity or LDL cholesterol oxidation at a dosage of about 30 mg/Kg
35 or less. The method may also be used to treat a

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condition that is associated with platelet activity or LDL cholesterol oxidation by administering a dietary supplement containing a flavonoid source and an enzyme that are effective for reducing platelet activity or LDL cholesterol oxidation.

In another aspect, the invention features an article of manufacture containing a dietary supplement that is effective for reducing platelet activity and LDL cholesterol oxidation contained within a packaging material wherein the packaging material is labeled to indicate that the dietary supplement is effective for reducing platelet activity or LDL cholesterol oxidation or both in a mammal at a dosage of about 30 mg/Kg or less. In another embodiment of the article of manufacture, the packaging material may be labeled to indicate that the dietary supplement is useful to treat a condition that is associated with platelet activity or LDL cholesterol oxidation.

Unless otherwise defined, all technical and scientific terms and abbreviations used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

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DESCRIPTION OF DRAWINGS

Figure 1 shows a schematic diagram describing the animal model for producing stenosed arteries to measure changes in coronary blood flow that are observed when
5 periodic thrombosis occurs in order to mimic the problems that occur in patients with narrowed coronary arteries due to atherosclerosis.

Figure 2 is a strip chart recording showing the repeated platelet-mediated thrombosis formation followed
10 by embolization, which produces the cyclical flow reductions (CFR's) that are observed for blood flow measured in stenosed arteries produced by the Folts model.

Figure 3 is a strip chart recording showing the
15 elimination of CFR's following ingestion of PROVEXCV™ due to the *in vivo* platelet inhibiting properties of PROVEXCV™, which is a preferred dietary supplement in accordance with the invention.

Figure 4 is a strip chart recording showing that
20 the CFR's eliminated by administering PROVEXCV™ did not reappear when epinephrine (adrenaline) was administered two and one half hours after administering PROVEXCV™.

Figure 5 shows a schematic diagram describing the whole blood aggregometry method used herein to measure
25 blood platelet activity *ex vivo*.

Figure 6 is a graph showing the time course of LDL cholesterol oxidation monitored at 234 nm in the presence of vitamin E, ProVex Plus™ or PROVEXCV™.

Figure 7 shows a graph of the level of protection
30 against LDL cholesterol oxidation provided by PROVEXCV™.

Figure 8 is a graph depicting the rate of LDL cholesterol oxidation.

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DETAILED DESCRIPTION

The present invention involves the discovery that the combination of certain flavonoids and enzymes in the form of a dietary supplement will reduce the dosage of supplement needed to effectively reduce platelet activity and LDL cholesterol oxidation in a mammal. The present invention further involves a method to treat conditions associated with platelet activity and LDL cholesterol oxidation by administering combinations of flavonoids, extracts and enzymes to reduce platelet activity and LDL cholesterol oxidation.

Coronary artery disease, cerebrovascular disease and peripheral vascular occlusions are characterized by arterial narrowing. If arterial narrowing is further compromised by thrombotic occlusions, blood flow is further decreased and myocardial infarction or stroke may occur. These conditions are associated with platelet activity and LDL cholesterol oxidation. Current treatments for these types of vascular occlusions, such as angioplasty, result in intimal and medial damage to arteries, which can result in restenosis, acute thrombosis, myocardial infarction and sudden coronary death in patients. Moreover, platelet-mediated thrombus formation also plays a role in unstable angina, myocardial infarction and restenosis following angioplasty or atherectomy. The present invention provides a dietary supplement and a method to prevent new and recurrent thrombotic occurrences by administering a dietary supplement that inhibits platelet activity and LDL cholesterol oxidation.

Methods to Evaluate Platelet Activity**1. The Folts Model**

The Folts coronary thrombosis model ("Folts model") is used to study platelet aggregation and

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thrombosis in a variety of animal models. Folts, J.D., Circulation (Supplement 4), 83:3-14 (1991); Folts, J.D., J. Cardiovasc. Drugs & Therapy, 9:31-43 (1995); Folts et al., Circulation, 54:365-370 (1976). The Folts model
5 mimics the problems that occur in patients with narrowed coronary arteries due to atherosclerosis.

To use the Folts model, the chest of a fully anesthetized animal is opened and the heart exposed. Although the experiments described herein involved dogs,
10 other animals amenable to the Folts model include pigs, rabbits, monkeys and other similar animals. Moreover, the Folts model is a model for the clinical syndrome of unstable angina and other platelet-mediated thrombotic events in humans. Samama et al., Thromb. Haemost.,
15 68:500-05 (1992); Raeder et al., Am. Heart J., 104:249-253 (1983); Sherry, S., Cardiovasc. Rev. Rep., 5:1208-19 (1984); Ikeda et al., J. Am. Coll. Cardiol., 21:1008-1017 (1993).

As is shown in Figure 1, the circumflex coronary
20 artery is dissected out and a coronary artery flow probe is placed on the artery. The coronary artery flow probe continuously measures blood flow through the artery without interfering with the artery. The artery is then clamped to create intimal and medial damage to the
25 coronary artery. A plastic constricting cylinder is placed around the outside of the coronary artery at the point of arterial damage to reduce the internal diameter of the coronary artery. The extent of stenosis or diameter reduction produced by the cylinder is altered
30 using an angioplasty balloon or other suitable method.

The coronary artery flow probe measures changes in the coronary artery blood flow when clots periodically develop. When an artery is stenosed, platelets
periodically aggregate in the narrowed and damaged part
35 of the coronary artery. Platelet aggregation produces a

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thrombosis (blood clot) that increases in size, and gradually cuts off the coronary artery blood flow. As the artery becomes occluded, pressure builds up behind the platelet aggregation and may dislodge the clot forcing it through the stenosis, which causes a sudden restoration of coronary blood flow in the narrowed artery. Platelet aggregation in a damaged artery that is sufficient to decrease blood flow is referred to as acute platelet thrombus formation (APTF).

Thrombotic arterial occlusion in human atherosclerotic arteries is thought to begin with the exposure of the thrombogenic surface beneath the intimal lining of an artery. In addition, current information suggests that a variety of different stimuli recruit platelets to adhere and aggregate at an exposed thrombogenic surface. It is to be understood, however, that the scope of the present invention is not limited by a particular theory of pathogenesis.

Repeated platelet-mediated thrombosis formation followed by embolization and the observed concomitant changes in blood flow are referred to as cyclical flow reductions (CFR's) or cyclical flow variations (CFV's). This document will use the term CFR's. CFR's have also been observed in damaged and narrowed arteries in humans with femoral artery or coronary artery disease. Folts et al., Tex. Heart Int. J., 9:19-27 (1982); Eichorn et al., J Am. Coll. Cardiol., 17:43-52 (1991). As Figure 2 shows, CFR's are detected and monitored by measuring coronary artery blood flow. Although Figure 2 is illustrative of CFR's observed for coronary blood flow, it is to be understood that the Folts method is applicable to arterial flow in general (e.g., femoral arterial flow). In addition, techniques needed to use the Folts model and variations of the model are described in Folts, J.D., Circulation (Supplement 4), 83:3-14

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(1991); Folts, J.D., J. Cardiovasc. Drugs & Therapy, 9:31-43 (1995) and the references therein.

The frequency or number of CFR's per unit of time is a direct measure of *in vivo* platelet activity. Folts, J.D., J. Cardiovasc. Drugs & Therapy, 9:31-43 (1995). By measuring the frequency and extent of CFR's in stenosed arteries, the Folts model can be used to evaluate agents that will alter platelet activity. Folts, J.D., J. Cardiovasc. Drugs Ther., 9:31-43 (1995). As such, the Folts model identifies platelet inhibitors, the extent of inhibitor activity, the effective dosages of inhibitors, the duration of inhibition and the ability of an inhibitor to counteract platelet agonists.

Aspirin provides an illustrative example of the Folts model. The Folts model demonstrated that aspirin inhibits platelet activity and eliminates CFR's under the experimental conditions. Folts et al., Clin. Res., 22:595 (1974) (abstract); Folts, J., Circulation (Supplement 4), 83:3-14 (1991); Folts, J.D., J. Cardiovasc. Drugs Ther., 9:31-43 (1995). Aspirin is known to inhibit platelet activity at a dosage of 5 mg/Kg. When platelet activity is inhibited with aspirin under conditions used in the Folts model, platelet activity can be renewed by increasing the concentration of epinephrine (adrenaline) or norepinephrine in the blood. This increase in adrenaline occurs naturally in many people when they are stressed, scared, anxious or exercising. Folts, J.D. & Rowe, G.G., Thromb. Res., 50:507-516 (1988). In addition, increasing the arterial stenosis will also cause a recurrence of CFR's abolished with aspirin. Folts, J., Circulation (Supplement 4), 83:3-14 (1991). As such, the Folts model provides an effective tool to evaluate inhibition of platelet activity.

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2. The Platelet Aggregometry Test

A second method to measure platelet activity in humans or animals is the whole blood platelet aggregometry test. Equipment and methods to perform whole blood platelet aggregometry evaluations described and used herein are available from Chronolog Inc., 2 West Park Rd., Haverton, PA 19083. To perform the test, a blood sample is drawn by standard methods, which is then placed in a plastic tube. A pair of electrodes is placed in the blood sample to measure the electrical resistance of the blood sample. The electrical resistance of blood is normally low due to the numerous ions and electrolytes found in blood. A known platelet aggregation stimulus, such as adenosine diphosphate (ADP) or collagen, is then added to the blood sample to activate the platelets. Activated platelets will adhere to the electrodes used in a platelet aggregometry test. As shown in Figure 5 (right side), the electrical resistance of the blood sample usually increases in a sigmoidal fashion as platelets aggregate on the electrodes. If a subject is administered a substance that inhibits platelet activity, the increase in resistance following the addition of a platelet stimulus will be smaller.

Aspirin provides an illustrative example of the use of the blood aggregometry test. A blood sample taken from a human will have a baseline level of platelet activity. Addition of ADP increases the resistance of the blood sample. See Figure 5, right side, "ADP" curve. A second blood sample is withdrawn two hours after the person is given a 325 mg tablet of aspirin. Platelet activation following the addition of ADP does not increase platelet activity to the level observed for the initial blood sample. See Figure 5, right side, "ADP+ASA" curve. The decrease in blood resistance observed is expressed as a percentage decrease in *ex vivo*

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platelet activity caused by aspirin. When epinephrine levels of a person, who has ingested aspirin, are raised prior to withdrawing a blood sample, the inhibitory effects observed for aspirin are diminished. See Figure 5, right side, "ADP+ASA+EPI" curve. Folts, J.D., J. Cardiovascular Drugs & Therapy, 9:31-43 (1995); Ingerman-Wojenski & Silver, Thromb. Haemost., 51:154-156 (1984).

Aspirin also responds similarly when collagen is used to stimulate platelet activity. When collagen is used to stimulate platelet activity in the blood aggregometry test, aspirin decreases platelet activity by about 30-40%. Therefore, the blood aggregometry technique may also be used as another measure of platelet inhibitory effectiveness produced by drugs or flavonoids.

3. Evaluating LDL cholesterol Oxidation

LDL cholesterol oxidation or protection therefrom can be determined by several methods. Kleinveld et al., Clin. Chem., 38(10):2066-2072 (1992) describes the methods for the LDL oxidation experiments described and used herein. A preferred way to practice the method encompasses extracting LDL cholesterol from whole blood samples collected from a subject using standard techniques. The extracted LDL cholesterol is divided into control and experimental samples. LDL oxidation is catalyzed by adding a copper ion solution to each LDL cholesterol sample. Copper ions are known to catalyze and enhance LDL cholesterol oxidation.

Control LDL cholesterol samples contain extracted LDL cholesterol and copper ions. The control samples provide a baseline measure of the antioxidants in the diet of the subject from whom the blood was drawn and in the LDL cholesterol prepared therefrom. Test LDL cholesterol samples are combined with copper ions in the

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presence or absence of additional compounds such as dietary supplements that may act as antioxidants.

Additionally, a subject may be administered an antioxidant by standard techniques prior to collecting a blood sample, which facilitates the measurement of antioxidant properties *in vivo*. To measure *in vivo* LDL cholesterol antioxidant properties, whole blood samples are obtained before and after administering an antioxidant source to be tested.

Copper ions accelerate the production of conjugated dienes in LDL cholesterol oxidation, which absorb light at 234 nm. Therefore, LDL cholesterol oxidation is monitored by following the increase in absorbance at 234 nm as a function of time as shown in Figure 6. Antioxidants prolong the onset of diene production in LDL cholesterol. As a result, LDL cholesterol samples containing antioxidants can be compared by computing the time elapsed before LDL cholesterol oxidation is observed (lag time). See Figure 7. The lag time is the best indicator of the LDL cholesterol protection from oxidative damage under the LDL oxidation experimental conditions described herein. Longer lag times denote better antioxidant properties.

Inhibiting Platelet Activity and Decreasing LDL

Cholesterol Oxidation

Many complex factors lead to atherosclerosis, coronary artery disease and related conditions. Among these factors, it is known that platelet interaction with arterial walls can enhance the atherosclerotic process. Furthermore, increased platelet activity is intimately involved in both the development of atherosclerosis and the transient and permanent occurrence of thrombotic events. Therefore, factors that reduce platelet activity on a daily basis should inhibit the development or

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occurrence of atherosclerosis, coronary artery disease and related conditions. Folts et al., J. Myocard. Ischemia, 6(8):33-40 (1994).

Epidemiological studies reveal a inverse
5 correlation between the consumption of foods high in antioxidant flavonoids or vitamin E with reduced coronary heart disease. Hertog et al., Lancet, 342(8878):1007-11 (1993); Waterhouse et al., Hypernutritious Foods, Agscience, Inc., Auburndale, Fl., 219-238 (1997). Food
10 sources known to contain flavonoids include red wine, beer, grape juice, fruits and vegetables. Extracts from grape seeds, grape skins, ginkgo biloba, bilberry and other similar fruits, vegetables and herbs are also flavonoid and antioxidant sources.

15 There are, however, hurdles that must be overcome to effectively obtain the benefits that are associated with flavonoids and antioxidants. One hurdle is the bioavailability of the active ingredients in dietary sources of flavonoids. When bioavailability or activity
20 is low, a person must consume large quantities of a platelet inhibitor or antioxidant dietary supplement to receive the benefits associated with a platelet inhibitor or antioxidant. As a result, low bioavailability may render a dietary supplement impractical, too costly or
25 both for many consumers.

In addition, the effectiveness of the platelet inhibition and antioxidant characteristics associated with individual flavonoids varies between particular flavonoids, e.g., flavonoids from grapes are better
30 platelet inhibitors in human volunteers and animals than flavonoids found in citrus fruit juices. Folts et al., J. Am. Coll. Cardiology, 29(2)(Supplement A):303A (1997); Folts, J.D., J. Am. Coll. Cardiology, 29(2)(Supplement A):226A (1997); Folts et al., J. Am. Coll. Cardiology,
35 29(2)(Supplement A):180A (1997).

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Moreover, the environmental conditions within the blood stream are not static. To be effective, a dietary supplement must be effective under a variety of conditions including various degrees of stress, excitement and exercise, which tend to elevate platelet activity levels.

Finally, many dietary sources of flavonoids also contain unwanted constituents such as the alcohol in wines and beers and the sugars and calories in grape juice. There are also toxicity concerns for various flavonoids and drugs. For example, aspirin has well documented side effects that include gastrointestinal irritation, loss of effectiveness in the presence of epinephrine and the inability to inhibit CFR's under extreme stenosis circumstances.

Therefore, it was hypothesized that it would be useful to create a dietary supplement that would inhibit platelet activity and LDL cholesterol oxidation under a variety of conditions in a practical and cost effective manner. It was further hypothesized that a key feature of a useful dietary supplement would be to increase the bioavailability of a dietary supplement by including constituents to enhance the absorption of the dietary supplement active ingredients. The present inventor has discovered such a supplement and the supplement is described and claimed herein.

In a first embodiment, the invention provides a dietary supplement containing at least one flavonoid source and an enzyme that are effective for inhibiting blood platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 30 mg/Kg or less. The dietary supplement may further contain flavonoid sources such as grape seed extracts, grape skin extracts, ginkgo biloba extracts, quercetin, bilberry extracts or any other specific flavonoid. The "flavonoid source" may be

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derived from any source and may include synthetic or purified flavonoids from known sources. The flavonoid source may also be, for example, one or more flavonoids determined to be highly active alone or as a combination
5 wherein the flavonoid is isolated from a complex mixture, such as a plant extract, containing numerous flavonoids.

While not bound by a particular theory of action, it is believed that the constituents of the presently disclosed dietary supplement act synergistically by
10 increasing bioavailability or pharmacologic interactions, to inhibit blood platelet activity and inhibit LDL cholesterol oxidation. In a related aspect of the invention, the dietary supplement remains effective for reducing platelet activity and inhibiting LDL cholesterol
15 oxidation in the presence of elevated platelet agonist (e.g., epinephrine) concentrations.

Examples of dietary supplement formulations may include one or more of the following constituents: fruit extracts, vegetable extracts, digestive enzymes, herbs,
20 flavonoids, antioxidants and other similar items.

Illustrative examples of useful digestive enzymes include pepsin, papain, fungal proteases, acid stable proteases, neutral stable proteases, alkaline stable proteases and bromelain.

25 Illustrative examples of useful flavonoids include catechins, procyanidins, proanthocyanidins, quercetin, rutin and glycosidic forms of the flavonoids.

Dietary supplements of the present invention may be delivered orally, intravenously, subcutaneously,
30 sublingually, intragastrically, as a phytosome or by any acceptable delivery method. In addition, the dietary supplement may be combined with any suitable carrier to facilitate delivery. Dietary supplements tested herein using the Folts model were administered either
35 intravenously or intragastrically. Absorption through the

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stomach lining usually takes 2-4 hours. As such, dietary supplements administered intragastrically may not affect CFR's until about 2-4 hours after administering a dietary supplement.

5 Commercial dietary supplements are generally formulated to be given orally. Useful forms of administration for dietary supplements include pills, pastes, powders, liquids and other common oral formulations. Preparations of the dietary supplements
10 may be manufactured using common manufacturing techniques for the various forms of the supplement described herein. The dietary supplements described herein additionally may contain magnesium stearate as a lubricant to facilitate the delivery the supplement into capsules. Magnesium
15 stearate is generally used at concentrations from 1-4 mg/capsule, preferably 1-2 mg/capsule.

The Folts model may be used to identify dietary supplement formulations that decrease platelet activity. A dog coronary artery is prepared to exhibit CFR's as
20 described herein. The dog is then administered measured doses of a dietary supplement by one of the approved methods. The effect that the dietary supplement dose has on the observed CFR's is monitored. By using the Folts model, the effective dose, half life and extent of
25 absorption can be determined. Blood samples taken from the dog provide information concerning supplement concentrations and platelet activity levels within the blood stream.

Additional experiments may be used to evaluate the
30 characteristics of useful dietary supplements. For example, the stenosis severity can be increased following administering a dietary supplement to determine if CFR's reoccur. In addition, platelet agonists administered at various time points preceding or following administering
35 a dietary supplement will identify dietary supplements

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that can sustain CFR elimination under platelet activating conditions. Useful agonists include epinephrine and norepinephrine. Useful doses of epinephrine for this purpose include epinephrine
5 administered intravenously at 0.2 μ g/Kg/min for 15 minutes.

Human volunteers or patients with human coronary artery disease may also be administered the dietary supplements described and claimed herein and variations
10 thereof to evaluate the efficacy of the dietary supplement using platelet aggregometry and LDL cholesterol oxidation experiments as described herein.

Useful dietary supplement formulations may include one or more of the following: grape seed extract, grape
15 skin extract, ginkgo biloba extract, bilberry extract, quercetin and an enzyme blend. The dietary supplement may be formulated by weight in the following manner: grape seed extract at 12% w/w, grape skin extract at 20% w/w, ginkgo biloba extract at 10% w/w, bilberry extract
20 at 10% w/w, quercetin at 24% w/w and an enzyme blend at 24% w/w.

As such, a useful dietary supplement containing flavonoids and enzymes in accordance with the present invention is PROVEXCV™. PROVEXCV™ is available from
25 Melaleuca, Inc., Idaho Falls, Idaho.

PROVEXCV™ contains the following ingredients per 380 milligram capsule:

<u>Ingredient</u>		<u>% Formulation</u>	<u>Amount</u>
30	Grape Seed Extract	12%	45 mg
	Ginkgo Biloba Extract	10%	38 mg
	Bilberry Extract	10%	38 mg
	Grape Skin Extract	20%	76 mg
	Quercetin	24%	92 mg
	Enzyme Blend	<u>24%</u>	<u>91 mg</u>
35	Total	100%	380 mg

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A supplement may also be prepared, in accordance with the present invention, to include all of the above ingredients except the Enzyme Blend.

The enzyme blend used in each 380 milligram capsule of PROVEXCV™ contains the following enzymes:

<u>Ingredient</u>	<u>% of Enzyme blend</u>	<u>Activity Units</u>
Fungal Protease 20053	25%	11,250 HUT
Fungal Protease 20054	25%	11,250 HUT
Acid Stable Protease	25%	3,375 SAPU
10 Bromelain	<u>25%</u>	5,760,000 PU
Total	100%	

The fungal proteases 20053 and 20054 are enzyme mixtures of acid, neutral and alkaline protease enzymes. HUT activity is the activity of an enzyme measured in the
15 FCC HUT assay, which is based on the hydrolysis of denatured hemoglobin. One HUT unit is defined as that amount of enzyme that produces a hydrosylate whose absorbance at 275 nm is equal to a solution of 1.10 mg/ml of tyrosine in 0.006 N HCl in one minute. SAPU activity
20 is measured in the FCC SAPU assay, which is based on hydrolysis of Hammarstan casein substrate. One SAPU unit is defined as that amount of enzyme that will liberate one μ mole of tyrosine per minute at pH 3 and 37 °C. PU activity is the activity of an enzyme measure in the FCC
25 PU assay, which is based on the hydrolysis of casein. One PU unit is defined as the amount of enzyme that liberates 1 μ g of tyrosine per hour at pH 6.0 and 40 °C. More information concerning the above referenced enzymes is available from the National Enzyme Company, Forsyth,
30 Missouri, (417)-546-4796 through technical bulletins concerning the enzymes.

The ingredients contained in PROVEXCV™ can be obtained from the following sources. The actual sources used are indicated by underlining the supplier.

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<u>Ingredient/Extract</u>	<u>Available from</u>
5 Grape Seed Extract	<u>Indena - Milan, Italy</u> Polyphenolics - Canandaigua, NY InterHealth - Concord, CA Tri-K Industries-Fanerson, NJ
10 Ginkgo Biloba Extract	<u>Indena - Milan, Italy</u> Weinstein Nutritional - Irvine, CA OptiPure - Los Angeles, CA Botanicals International - Long Beach, CA
Bilberry Extract	<u>Indena - Milan, Italy</u> OptiPure - Los Angeles, CA Chemco Industries - Los Angeles, CA
15 Grape Skin Extract	<u>Freeman Industries - Tuckahoe, NY</u> Weinstein Nutritional - Irvine, CA Brucia Extracts - California
20 Quercetin	<u>Weinstein Nutritional - Irvine, CA</u> Botanicals International - Long Beach, CA Triarco Industries - Wayne, NY
Enzyme Blend	<u>National Enzyme Co. - Forsyth, MO</u> MakWood - Thiensville, WI Botanicals International - Long Beach, CA
25 Citrus Extract	<u>Botanicals International - Long Beach, CA</u>

ProVex Plus™ contains the following ingredients per 125 milligram capsule:

<u>Ingredient</u>	<u>% Formulation</u>	<u>Amount</u>
30 Grape Seed Extract	20%	25 mg
Ginkgo Biloba Extract	8%	10 mg
Bilberry Extract	8%	10 mg
Grape Skin Extract	24%	30 mg
Citrus Extract	40%	50 mg
35 Total	100%	125 mg

Experiments in the Folts model using a supplement containing all of the ingredients of PROVEXCV™ except the enzyme blend have shown that such a supplement is effective for eliminating CFR's at a dosage of about 20

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mg/Kg or less. It has been discovered that the dosage needed to eliminate CFR's observed in the Folts model can be further reduced by combining a dietary flavonoid with an enzyme. The addition of an enzyme blend containing
5 equal parts of two fungal proteases, an acid stable protease and bromelain to a supplement containing all of the ingredients of PROVEXCV™ except the enzyme blend decreased the dosage needed to eliminate CFR's in the animal model used herein from about 20 mg/Kg to about 10
10 mg/Kg.

The effectiveness of PROVEXCV™ was such that the PROVEXCV™ decreased the dosage needed to eliminate CFR's in the animal model used herein from about 30 mg/Kg needed for ProVex Plus™ to about 10 mg/Kg for PROVEXCV™.
15 As a result, useful doses of dietary supplements described herein are about 30 mg/Kg or less. Preferably, an effective dosage is about 20 mg/Kg or less. More preferably, an effective dosage is about 10 mg/Kg or less.

20 As such, the consumption of PROVEXCV™ with its antioxidant and platelet inhibitory properties may protect against the development of coronary artery disease, acute occlusive thrombosis, death from myocardial infarction and other conditions that are
25 associated with platelet activity and LDL cholesterol oxidation.

Another embodiment of the invention includes a dietary supplement designated PROVEXCV2™. Like ProVex Plus™ and PROVEXCV™, PROVEXCV2™ can be used as a dietary
30 supplement to inhibit platelet activity or LDL cholesterol oxidation. PROVEXCV2™ contains the following ingredients per 1,638 milligrams:

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<u>Ingredient</u>	<u>% Formulation</u>	<u>Amount</u>
Enzyme Blend	4.58%	75 mg
Grape Seed Extract	3.30%	54 mg
Grape Skin Extract	67.77%	1,110 mg
5 Quercetin	7.32%	120 mg
Ginkgo Biloba Extract	9.71%	159 mg
Bilberry Extract	<u>7.32%</u>	<u>120 mg</u>
Total	100%	1,638 mg

The ingredients for the dietary supplements described herein, including PROVEXCV2™, can be obtained from any supplier. For example, the suppliers listed above can be used to obtain all the ingredient for PROVEXCV2™. In addition, the ingredients can be obtained from sources that were not subjected to a fermentation process. For example, unfermented grape seed extract and unfermented grape skin extract can be used as ingredients for the dietary supplements described herein. Such unfermented ingredients can be obtained from any supplier such as Polyphenolics (Canandaigua, NY).

Briefly, fermentation processes are used during the production of wine. If an ingredient is obtained from a supplier involved in wine production, then the ingredient could be a fermented ingredient. For example, a fermented ingredient, such as fermented grape seed extract or fermented grape skin extract, can be any ingredient isolated from a source, such as grapes, that were subjected to fermentation. In contrast, an unfermented ingredient can be any ingredient isolated from a source not subjected to fermentation. Such unfermented ingredients can be more effective sources of flavonoids than fermented ingredients. For example, unfermented grape seed extract or unfermented grape skin extract can inhibit platelet activity or LDL cholesterol oxidation more effectively than fermented grape seed extract or fermented grape skin extract.

It is noted that the percentage for each ingredient in ProVex Plus™, PROVEXCV™, and PROVEXCV2™ can

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be changed, provided the resulting composition can inhibit platelet activity or LDL cholesterol oxidation. For example, the percentage of ginkgo biloba extract can be increased to greater than 10%.

5 In another aspect, the invention provides a method to inhibit platelet activity or LDL cholesterol oxidation in a mammal by administering a dietary supplement containing at least one flavonoid source and an enzyme that are effective for reducing platelet activity or LDL
10 cholesterol oxidation or both.

In another aspect of the invention, a method to treat a condition associated with platelet activity or LDL cholesterol oxidation in a mammal is provided. The method involves the step of administering a dietary
15 supplement containing at least one flavonoid source and an enzyme that are effective for reducing platelet activity or LDL cholesterol oxidation at a dosage of about 30 mg/Kg or less.

To practice the described methods, a mammal is
20 given a dose of a dietary supplement as described herein by an acceptable delivery method. The dose can be administered hourly, daily, weekly or a fraction thereof depending on the circumstances. After administration of the dietary supplement a mammal can be evaluated for
25 platelet activity and LDL cholesterol oxidation. The platelet activity and LDL cholesterol oxidation levels can then be compared to the same levels prior to administering the dietary supplement.

In another aspect of the invention, an article of
30 manufacture containing a dietary supplement that is effective for reducing platelet activity and LDL cholesterol oxidation is provided. The article is contained within a packaging material that is labeled to indicate that the dietary supplement is useful for
35 reducing platelet activity or LDL cholesterol oxidation

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or both in a mammal at a dosage of about 30 mg/Kg or less. In another embodiment of the article of manufacture, the packaging material can be labeled to indicate that the dietary supplement is useful to treat a
5 condition that is associated with platelet activity or LDL cholesterol oxidation.

Any common packaging and printing method can be used to prepare the article of manufacture.

The invention will be further understood with
10 reference to the following illustrative embodiments, which are purely exemplary, and should not be taken as limiting the true scope of the present invention as described in the claims.

EXAMPLES

15 Example 1 - Evaluating Platelet Inhibition by Dietary Supplements

Ten anesthetized dogs with coronary stenosis and medial damage were prepared for evaluation using the methods of the Folts model as disclosed in Folts, J.,
20 Circulation (Supplement 4), 83:3-14 (1991). Blood pressure and coronary artery blood flow were monitored continuously throughout the experiment. As exemplified in Figure 2, CFR's similar to the CFR's shown in Figure 2, which were due to acute platelet thrombus formation
25 (APTF), occurred 8 ± 3 times in a 30 min interval in 10 dogs prior to administering PROVEXCV™. All figures showing strip chart recordings are on the same scale, which is 10 minutes per twelve squares. 10 mg/Kg of PROVEXCV™ was then administered by stomach tube to each
30 dog. As exemplified in Figure 3, gastric administration of 10 mg/Kg of PROVEXCV™ eliminated the observed CFR's in 174 ± 24 min in 9 of the 10 dogs. The CFR's observed in the tenth dog were reduced to 2 CFR's in a 30 minute interval three hours after administering 10 mg/Kg

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PROVEXCV™. There was no change in heart rate or arterial blood pressure produced by the dietary flavonoids in PROVEXCV™.

When evaluated in the Folts model, platelet activity that is inhibited by aspirin can be reactivated by administering 0.2 µg/Kg/min epinephrine intravenously. Folts, J., Circulation (Supplement 4), 83:3-14 (1991). Eight dogs that had CFR's eliminated by administering 10 mg/Kg PROVEXCV™ were administered 0.2 µg/Kg/min epinephrine intravenously for 15 minutes. The right side of Figure 4 shows that CFR's eliminated by gastric administration of 10 mg/Kg PROVEXCV™ did not reappear when epinephrine was administered at 0.2 µg/Kg/min intravenously for 15 minutes in all 8 dogs.

Ex vivo platelet activity was determined for all ten dogs using the whole blood aggregometry method as described herein. Blood samples were taken from each dog before and after the administration of 10 mg/Kg of PROVEXCV™ and tested. When platelet aggregation was stimulated by collagen, ex vivo whole blood platelet aggregation was decreased by 40% ± 9% in all 10 dogs administered 10 mg/Kg PROVEXCV™. Unlike aspirin as shown in Figure 5, the observed platelet aggregation increases in response to epinephrine were not observed with PROVEXCV™.

Example 2 - Evaluating LDL cholesterol Oxidation of Dietary Supplements

As described above, LDL cholesterol oxidation is commonly measured by observing absorbance at 234 nm (Abs_{234nm}) as a function of time while the LDL cholesterol is exposed to oxidative conditions. The antioxidant effectiveness of PROVEXCV™ and other substances was determined.

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LDL cholesterol was prepared from human volunteer blood samples. Isolated LDL cholesterol was then mixed with a buffer, vitamin E, ProVex Plus™ ("Orig" in Figure 6) or PROVEXCV™ ("Curr" in Figure 6). Experimental solutions of ProVex Plus™ and PROVEXCV™ were prepared at 0.5 and 1.0 mg/L final concentration. The concentrations chosen for ProVex Plus™ and PROVEXCV™ were an estimate of expected blood levels of the dietary supplements based on accepted blood absorption models. To each sample a measured amount of copper ions was added. The amount of vitamin E used was comparable to the amount anticipated to be in the blood of a person administered 400 IU of vitamin E.

After mixing, the Abs_{234nm} of each solution was then monitored as a function of time. As shown in Figure 6, incubating LDL cholesterol with ProVex Plus™ or PROVEXCV™ at 0.5 mg/L or 1.0 mg/L protected LDL cholesterol from oxidation longer than that observed for a well known antioxidant vitamin E at 5.3 IU/L.

As shown in Figure 7, LDL cholesterol did not demonstrate appreciable oxidation until 45 minutes following the addition of copper ions. Vitamin E at the described concentration protected LDL cholesterol from oxidation for about 95 minutes. 1.0 mg/L of ProVex Plus™ protected LDL cholesterol against oxidation for more than 100 minutes. Finally, 1.0 mg/L of PROVEXCV™ protected LDL cholesterol against oxidation for more than 150 minutes under these experimental conditions. As such, ProVex Plus™ and PROVEXCV™ are better antioxidants than vitamin E.

Example 3 - Evaluating PROVEXCV2™

In dogs, the effects of PROVEXCV2™ on platelet activity was evaluated in vivo using the Folts model and ex vivo using the whole blood platelet aggregometry test.

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Briefly, ten adult mongrel dogs of either sex were anesthetized and the chest opened at the fifth intercostal space. The left circumflex coronary artery was dissected out and an electromagnetic flow probe
5 placed around the artery to measure coronary blood flow. Distal to the flow probe, the artery was clamped three times with a special surgical clamp to produce intimal and medical damage. A plastic cylinder of appropriate inside diameter was placed around the artery to produce a
10 70% reduction in arterial diameter.

During the occurrence of CFR's, a blood sample was drawn for the ex vivo the whole blood platelet aggregometry test. After monitoring consistent formation of CFR's for 20 minutes, 15 mg/kg of PROVEXCV2™ was
15 administered by a stomach tube. The CFR's were monitored for 3 hours. When the CFR's were abolished, a second blood sample was drawn to repeat the ex vivo platelet aggregation test. Epinephrine (0.2 µg/kg/min) was then infused for 20 minutes to observe whether the CFR's could
20 be renewed.

The ten dogs with coronary artery stenosis and intimal damage had CFR's, due to acute platelet thrombus formation, at a frequency of 7 ± 3 per 30 minutes. After 15 mg/kg of PROVEXCV2™ was given by gastric tube, the
25 CFR's were abolished for 164 ± 29 minutes. The intravenous infusion of epinephrine (0.2 µg/kg/min for 20 minutes) did not renew the CFR's in any dog. Again, epinephrine infusion after the abolishment of CFR's with 5-10 mg/kg of aspirin can result in renewed CFR's in 50-60% of dogs
30 studied. Thus, PROVEXCV2™ appears to inhibit platelet activity more potently than aspirin.

The activity of platelets in whole blood samples collected before and after PROVEXCV2™ administration were evaluated ex vivo using the whole blood platelet
35 aggregometry test. After PROVEXCV2™ administration,

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platelet aggregation, induced by ADP (20 μ mol/ml), decreased by $42 \pm 10\%$ ($p < 0.03$). In addition, the platelet aggregation produced by the combination of epinephrine and ADP was decreased by $32 \pm 11\%$ ($p < 0.05$) after PROVEXCV2™ administration.

In humans, the effects of PROVEXCV2 on platelet activity also was evaluated *ex vivo* using the whole blood platelet aggregometry test. Specifically, twelve healthy human volunteers (8 men, 4 women) aged 22-50 years of age were recruited. They abstained from tea, alcoholic beverages, grape products, flavonoid and vitamin supplements, and all medications including aspirin products for one week prior to and throughout the study. Vegetarians were excluded from the study.

On day 1 of the study for each volunteer, 18 ml of venous blood was drawn for the *ex vivo* whole blood platelet aggregometry test, between 8 am and 12 noon while in the fasting state. Then, each volunteer was instructed to consume 5-7 capsules (about 20/mg/kg/day), depending on their body weight, of PROVEXCV2™ for 7-14 days. After 7-14 days, each volunteer returned to the laboratory in the fasting state, but having taken that day's dose of PROVEXCV2™, and had a second blood sample drawn. This sample was drawn about 2-4 hours after the last dose of PROVEXCV2™.

Briefly, 18 ml of whole blood was drawn into a syringe containing 2 ml of 3.8% sodium citrate as an anticoagulant. The blood was immediately diluted with an equal volume of preservative-free saline. One milliliter (1 ml) of the diluted blood was placed in a cuvette with a siliconized stir bar and warmed to 37°C for 5 minutes. An electrode is placed in the cuvette to measure the impedance change, which is proportional to the platelet aggregation. Once the baseline platelet activity was recorded, a high dose of collagen (12.5 μ g) was added to

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the pre-warmed blood and placed in the aggregometer to obtain a maximum platelet aggregation response. The change in impedance produced by platelet aggregation was followed for 7 minutes. Sub-maximal doses of collagen
5 (0.5 $\mu\text{g/ml}$), phorbol 12-myristate 13-acetate (PMA) (0.5 nmol/ml), and ADP (20 $\mu\text{mol/ml}$) were used as platelet agonists. In addition, ADP (20 $\mu\text{mol/ml}$) was also used as an agonist to aggregate platelets in 1 ml of blood pre-incubated for 1 minute with epinephrine (0.5 $\mu\text{g/ml}$). The
10 aggregation responses were measured in duplicate in the control sample and compared to the responses after 7-14 days of daily PROVEXCV2™.

The platelet aggregation response to collagen (0.5 $\mu\text{g/ml}$), ADP (20 $\mu\text{mol/ml}$), and a phorbol ester,
15 phorbol 12-myristate 13-acetate (PMA; 0.5 nmol/ml), in the samples collected after 7-14 days of daily PROVEXCV2™ administration revealed a reduction of 51.6 \pm 41.1% ($p<0.005$), 39.8 \pm 41.5% ($p<0.005$), and 17.9 \pm 10.0% ($p<0.002$), respectively. In addition, the platelet
20 aggregation in response to a combination of epinephrine (0.5 $\mu\text{g/ml}$) and ADP (20 $\mu\text{mol/ml}$) in the samples collected after 7-14 days of daily PROVEXCV2™ administration revealed a reduction of 14.9 \pm 8.5% ($p<0.05$). These results suggest that the mechanism of platelet activity
25 inhibition by PROVEXCV2™ may be through the inhibition of platelet protein kinase C since PMA induced platelet aggregation was reduced after 7-14 days of daily PROVEXCV2™ administration.

In addition to evaluating the effects of
30 PROVEXCV2™ on platelet activity, the effects of PROVEXCV2™ on LDL cholesterol oxidation was studied. Briefly, LDL was isolated from serum collected from a healthy-fasting volunteer using sequential ultra-centrifugation with spins at densities of 1.006 g/ml to
35 remove VLDL/chylomicrons and 1.063 g/ml (KBr) to recover

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the LDL from the denser HDL and serum proteins. The duration of each spin was 3 hours using a Beckman Optima set at 100,000 rpms ($>4,000,000 \times g$). The LDL was dialyzed against phosphate buffered saline (PBS) containing EDTA for 48 hours with several changes of the buffer. The dialyzed material was then checked by electrophoresis (agarose gel) to demonstrate the absence of other lipoproteins fractions or serum proteins. The protein concentration of the isolated LDL was measured and adjusted to 0.5 g/L using EDTA-containing PBS for dilutions. The LDL solution was then aliquoted in 0.7 ml volumes into small glass containers with screw caps enclosures. The vials and the LDL solutions were flushed with nitrogen to remove traces of oxygen to ensure stability and stored at -80°C until just prior to use.

LDL oxidation was performed as described above. Briefly, the LDL was diluted with EDTA-free PBS by mixing 100 μl of thawed LDL with 900 μl of PBS just prior to the oxidizability study. Ten (10) μl of freshly prepared CuCl_2 (final concentration of 5 $\mu\text{mol/L}$) was added to the LDL solution to initiate oxidation. The rate of formation of conjugated dienes was monitored spectrophotometrically at 234 nm at 30°C for 5 hours taking absorbance readings every 3 minutes. The spectrophotometer is equipped with a six place automatic sample holder to provide a maximum of six specimens analyzed in a single run. LDL alone with PBS and CuCl_2 is used as a control when assessing the ability of PROVEXCV2TM using direct mixing *in vitro* experiments.

The time course of LDL oxidation shows three consecutive phases: a lag phase in which there is hardly any conjugated diene formation, a propagation phase with a rapid increase in diene formation, and finally a decomposition phase. The lag phase, defined as the time period between the addition of CuCl_2 and the start of

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propagation, is considered to reflect the LDL's susceptibility to oxidative stress.

The rate of production of conjugated dienes in a sample of LDL cholesterol is shown in Figure 8. Native
5 LDL cholesterol was compared to an equal sample of LDL cholesterol incubated with Vitamin E (5.3 mg/L) or with PROVEXCV2™ (1.0 mg/L). The lag time before oxidation begins was delayed by vitamin E and delayed to a significantly greater extent by PROVEXCV2™. This result
10 indicates that PROVEXCV2™ is a more potent antioxidant than Vitamin E, when exposed to copper ion enhanced oxidation.

Other aspects, advantages, and modifications are within the scope of the following claims.

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WHAT IS CLAIMED IS:

1. A dietary supplement comprising at least one flavonoid source and an enzyme, said supplement effective for inhibiting platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 30 mg/Kg or less.
2. The supplement of claim 1, wherein said flavonoid source comprises one or more of grape seed extract, grape skin extract, bilberry extract, ginkgo biloba extract or quercetin.
3. The supplement of claim 1, wherein said enzyme comprises one or more of a fungal protease, acid stable protease, or bromelain.
4. The supplement of claim 1, wherein said supplement is effective for inhibiting platelet activity and LDL cholesterol oxidation at a dosage of about 20 mg/Kg or less.
5. The supplement of claim 1, wherein said supplement is effective for inhibiting platelet activity and LDL cholesterol oxidation at a dosage of about 10 mg/Kg or less.
6. The supplement of claim 1, wherein said flavonoid source comprises at least one grape extract.
7. The supplement of claim 1, wherein said flavonoid source comprises bilberry extract.
8. The supplement of claim 1, wherein said flavonoid source comprises ginkgo biloba extract.

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9. The supplement of claim 1, wherein said flavonoid source comprises quercetin.

10. The supplement of claim 1, wherein said supplement is effective for inhibiting blood platelet activity for
5 at least four hours following ingestion of said supplement.

11. The supplement of claim 1, wherein said supplement is effective for inhibiting platelet activity in a mammal at a dosage of about 30 mg/Kg or less and following
10 administration of epinephrine at a dosage of about 0.2 μ g/Kg/min.

12. The supplement of claim 1, wherein said enzyme comprises about 24% or less of said supplement by weight.

13. The supplement of claim 1, wherein said dietary
15 supplement comprises PROVEXCV™.

14. The supplement of claim 1, wherein said dietary supplement comprises PROVEXCV2™.

15. A dietary supplement comprising a grape seed extract, grape skin extract, ginkgo biloba extract,
20 bilberry extract, quercetin, fungal protease, acid stable protease and bromelain, said supplement effective for inhibiting platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 30 mg/Kg or less.

25 16. The supplement of claim 15, said supplement being in the form of a pill.

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17. The supplement of claim 15, said supplement being in the form of a powder.

18. The supplement of claim 15, said supplement being in liquid form.

5 19. The supplement of claim 15, said supplement being in the form of a phytosome.

20. The supplement of claim 15, wherein said supplement is effective for inhibiting platelet activity and LDL cholesterol oxidation at a dosage of about 20
10 mg/Kg or less.

21. The supplement of claim 15, wherein said supplement is effective for inhibiting platelet activity and LDL cholesterol oxidation at a dosage of about 10 mg/Kg or less.

15 22. A dietary supplement comprising an unfermented flavonoid source and an enzyme, said supplement effective for inhibiting platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 30 mg/Kg or less.

20 23. The supplement of claim 22, wherein said supplement comprises a plurality of flavonoid sources.

24. The supplement of claim 23, wherein said supplement comprises PROVEXCV2™.

25 25. A method to inhibit platelet activity or LDL cholesterol oxidation in a mammal comprising administering a dietary supplement comprising at least one flavonoid source and an enzyme, said supplement

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effective for inhibiting platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 30 mg/Kg or less.

26. The method of claim 25, wherein said dietary
5 supplement reduces platelet activity and LDL cholesterol oxidation at a dosage of about 20 mg/Kg or less.

27. The method of claim 25, wherein said dietary supplement reduces platelet activity and LDL cholesterol oxidation at a dosage of about 10 mg/Kg or less.

10 28. The method of claim 25, wherein said dietary supplement reduces platelet activity and LDL cholesterol oxidation.

29. A method to treat a condition associated with platelet activity or LDL cholesterol oxidation comprising
15 administering a dietary supplement comprising at least one flavonoid source and an enzyme, said supplement effective for inhibiting platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 30 mg/Kg or less.

20 30. An article of manufacture comprising a dietary supplement comprising at least one flavonoid source and an enzyme, said supplement effective for inhibiting platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 30 mg/Kg or less contained
25 within a packaging material wherein said packaging material is labeled to indicate that said dietary supplement is useful for reducing platelet activity or LDL cholesterol oxidation or both.

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31. The article of manufacture of claim 30, wherein said packaging material is labeled to indicate that said dietary supplement is useful for treating a condition that is associated with platelet activity or LDL
5 cholesterol oxidation or both.

32. A dietary supplement comprising at least one flavonoid source, said supplement effective for inhibiting platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 30 mg/Kg or
10 less.

33. The supplement of claim 32, wherein said supplement is effective for inhibiting platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 20 mg/Kg.

15 34. The supplement of claim 32, further comprising an enzyme.

35. A dietary supplement comprising an unfermented flavonoid source, said supplement effective for inhibiting platelet activity and LDL cholesterol
20 oxidation in a mammal at a dosage of about 30 mg/Kg or less.

36. The supplement of claim 35, wherein said supplement is effective for inhibiting platelet activity and LDL cholesterol oxidation in a mammal at a dosage of
25 about 20 mg/Kg.

37. The supplement of claim 35, further comprising an enzyme.

ANIMAL MODEL FOR PRODUCING CONDITIONS SIMILAR TO A
PATIENT CORONARY ARTERY DISEASE

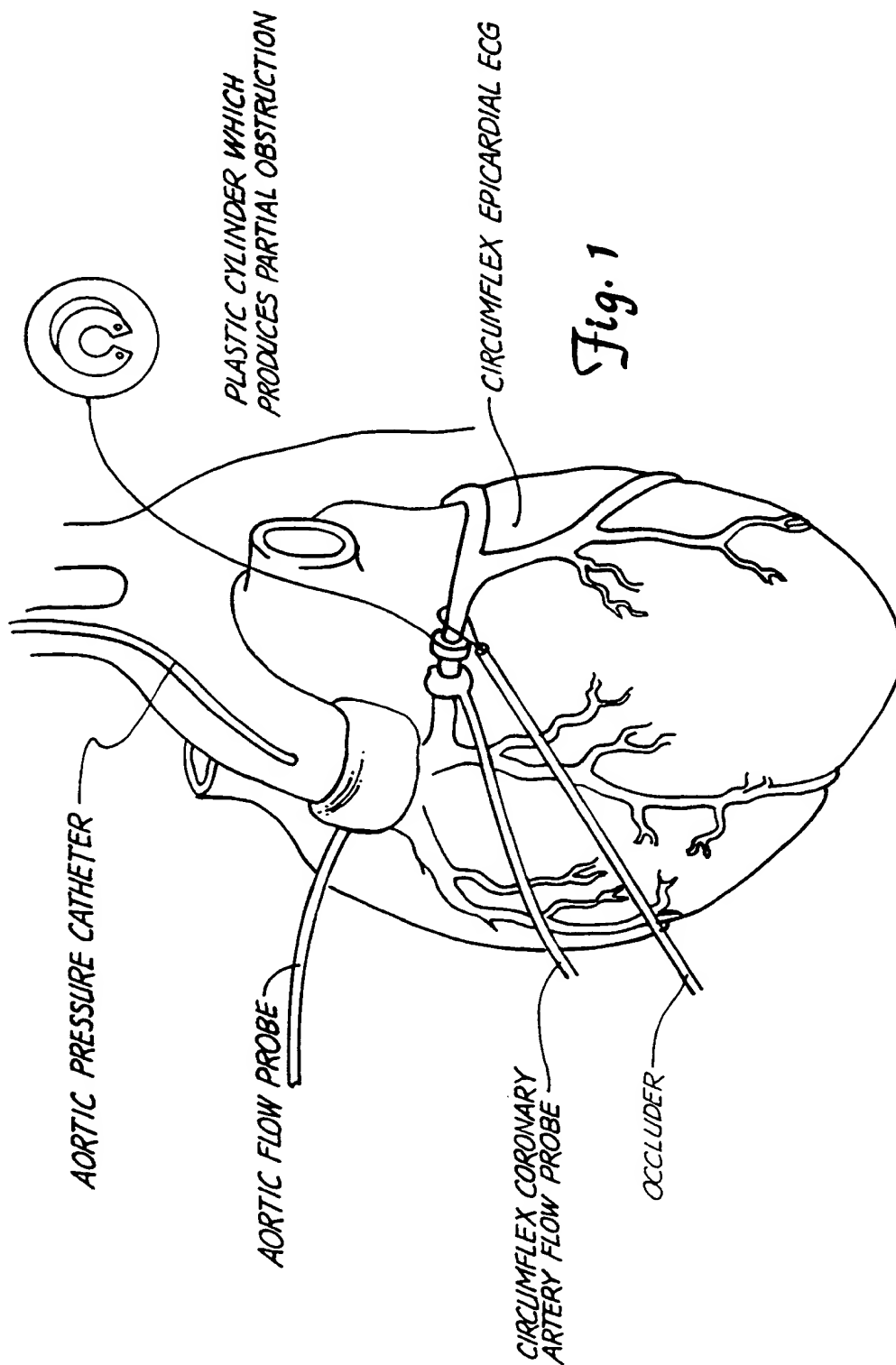
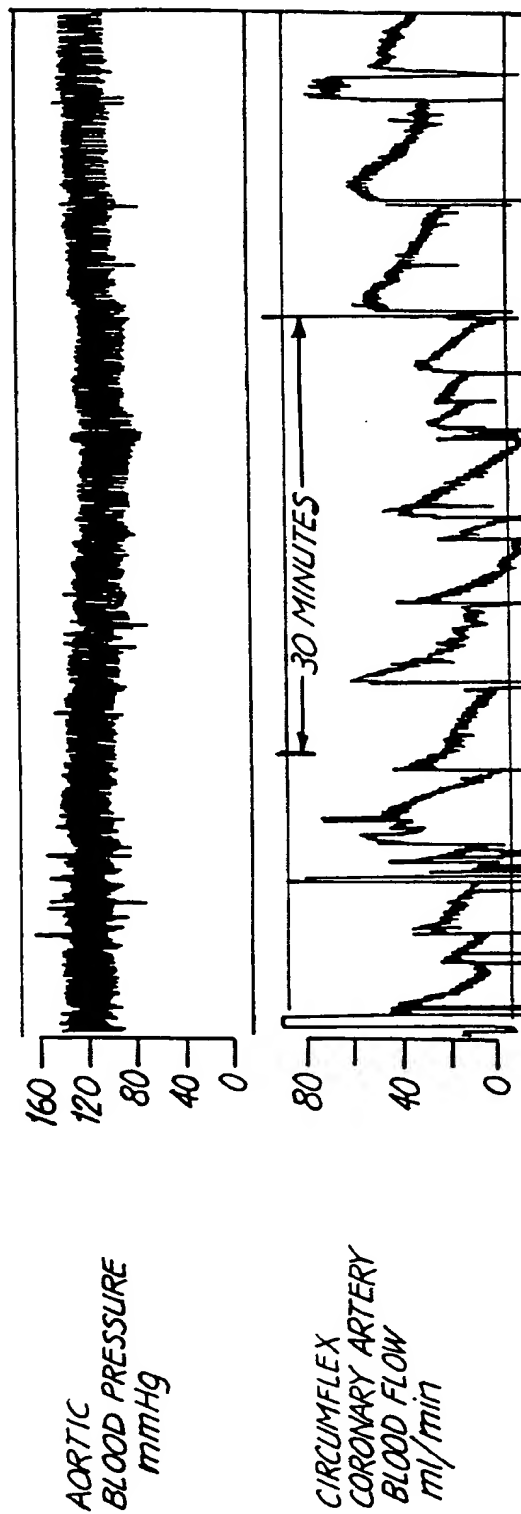


Fig. 1



10mg/kg PROVEX CV™
BY STOMACH TUBE

Fig. 2

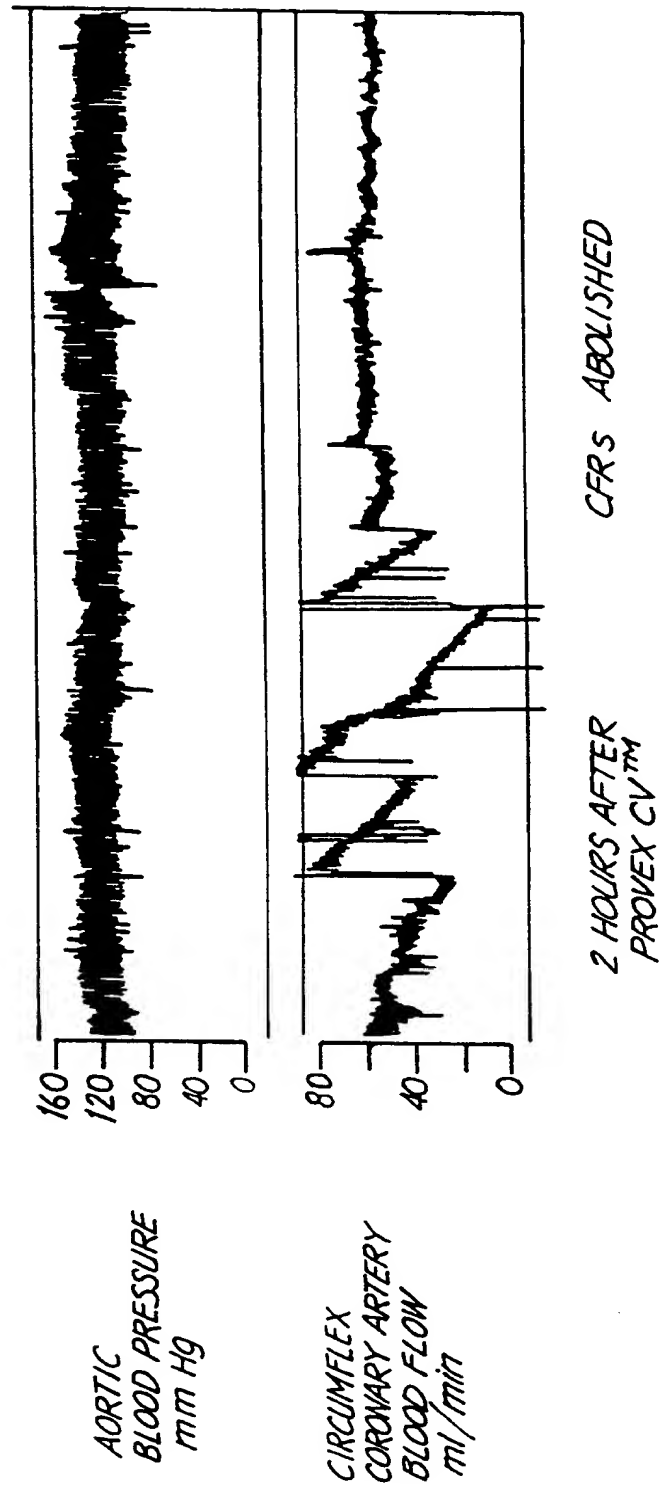


Fig. 3

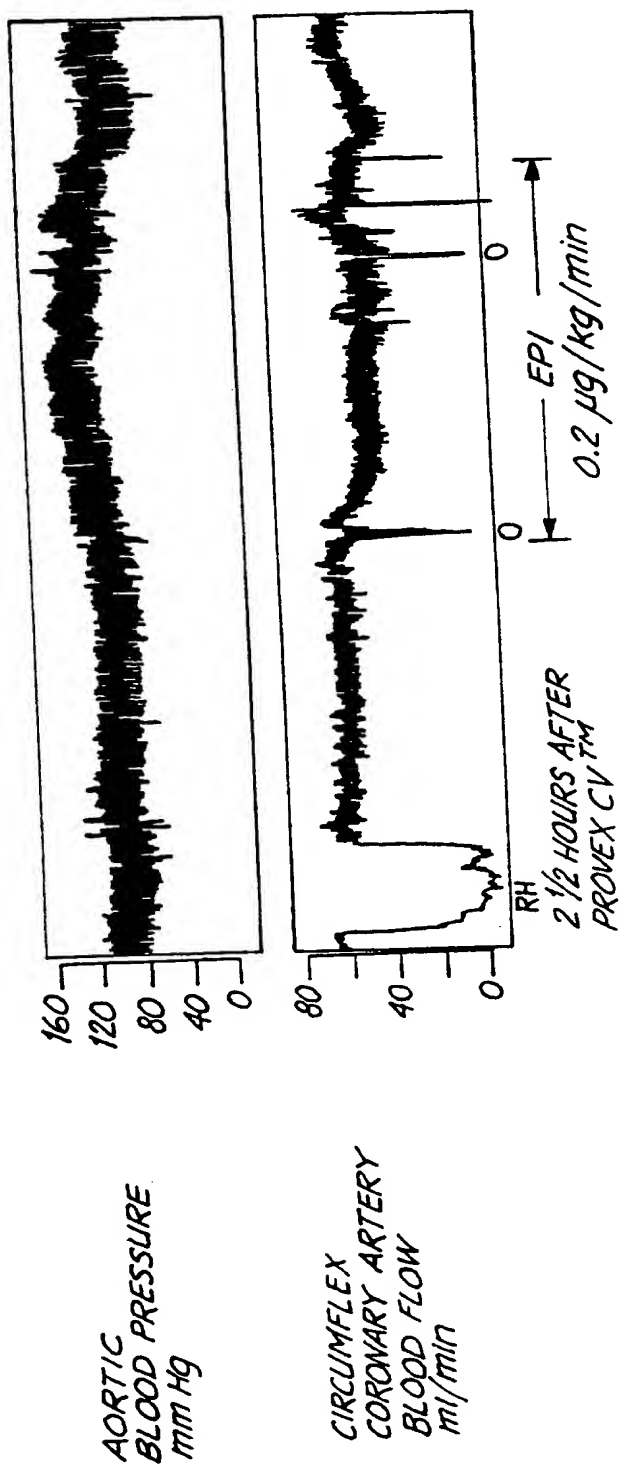


Fig. 4

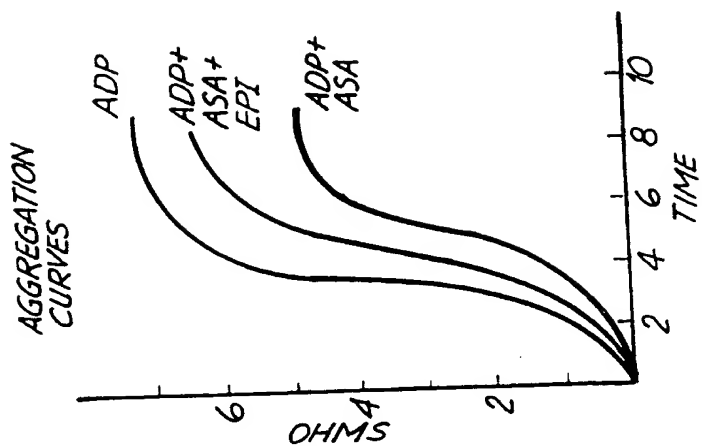


Fig. 5

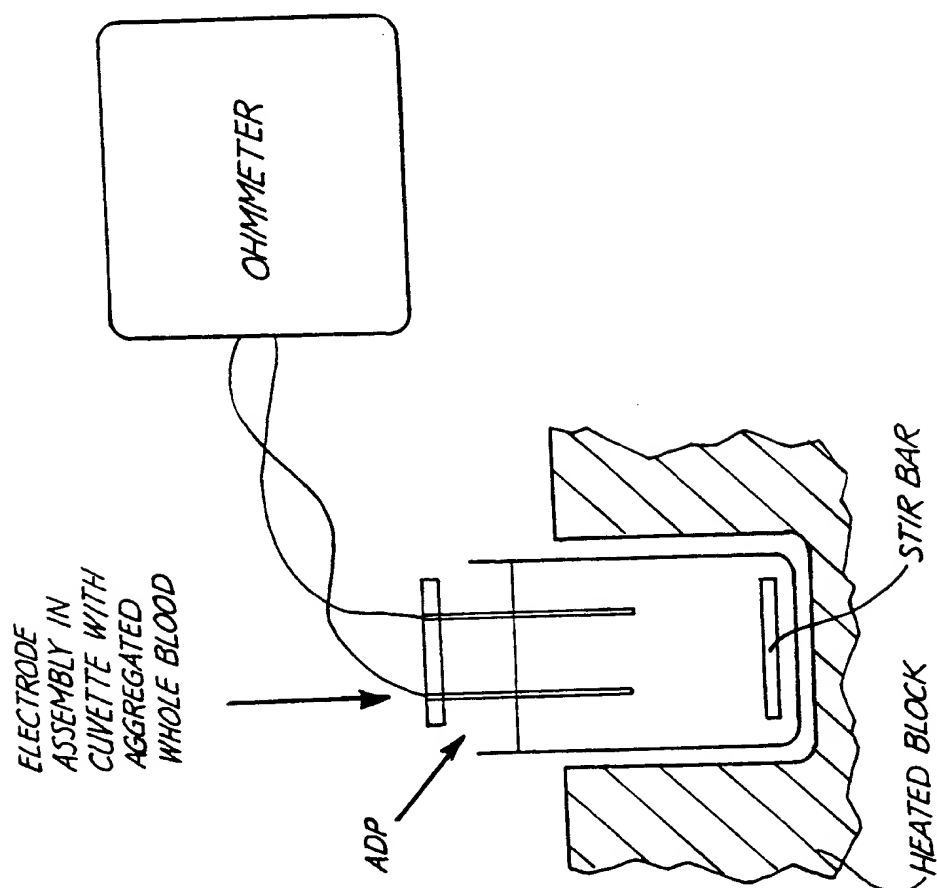
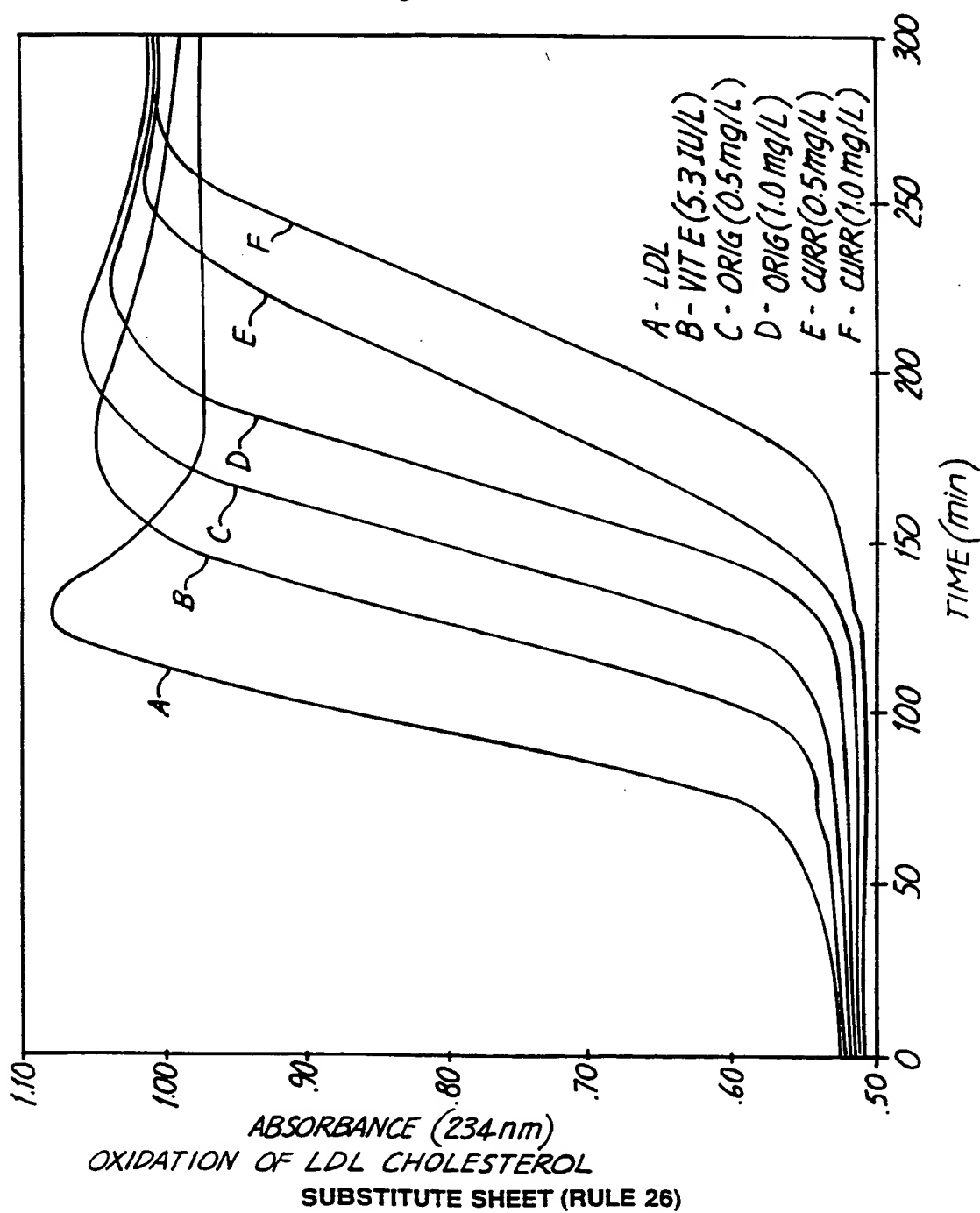


Fig. 6



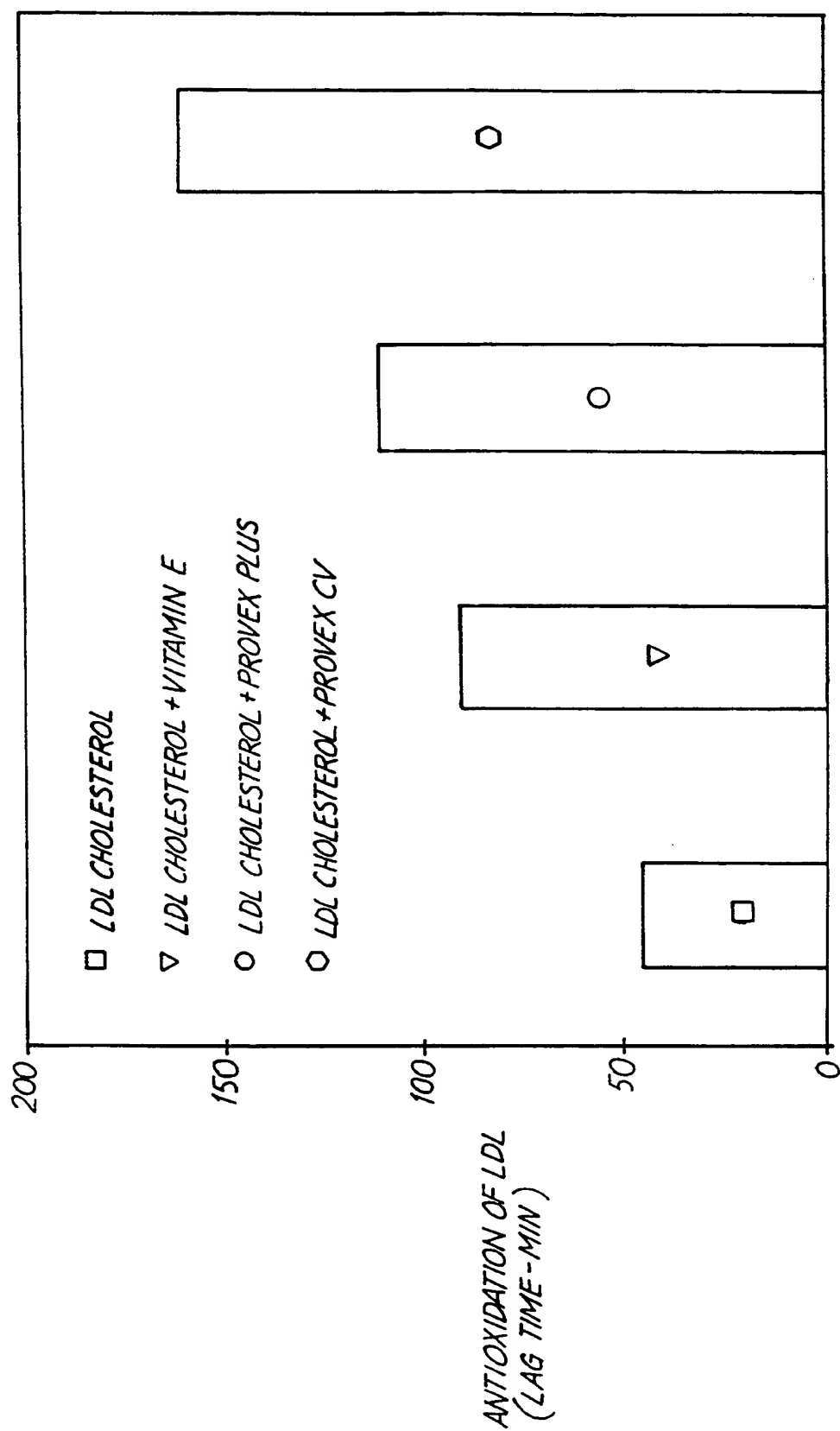


Fig. 7

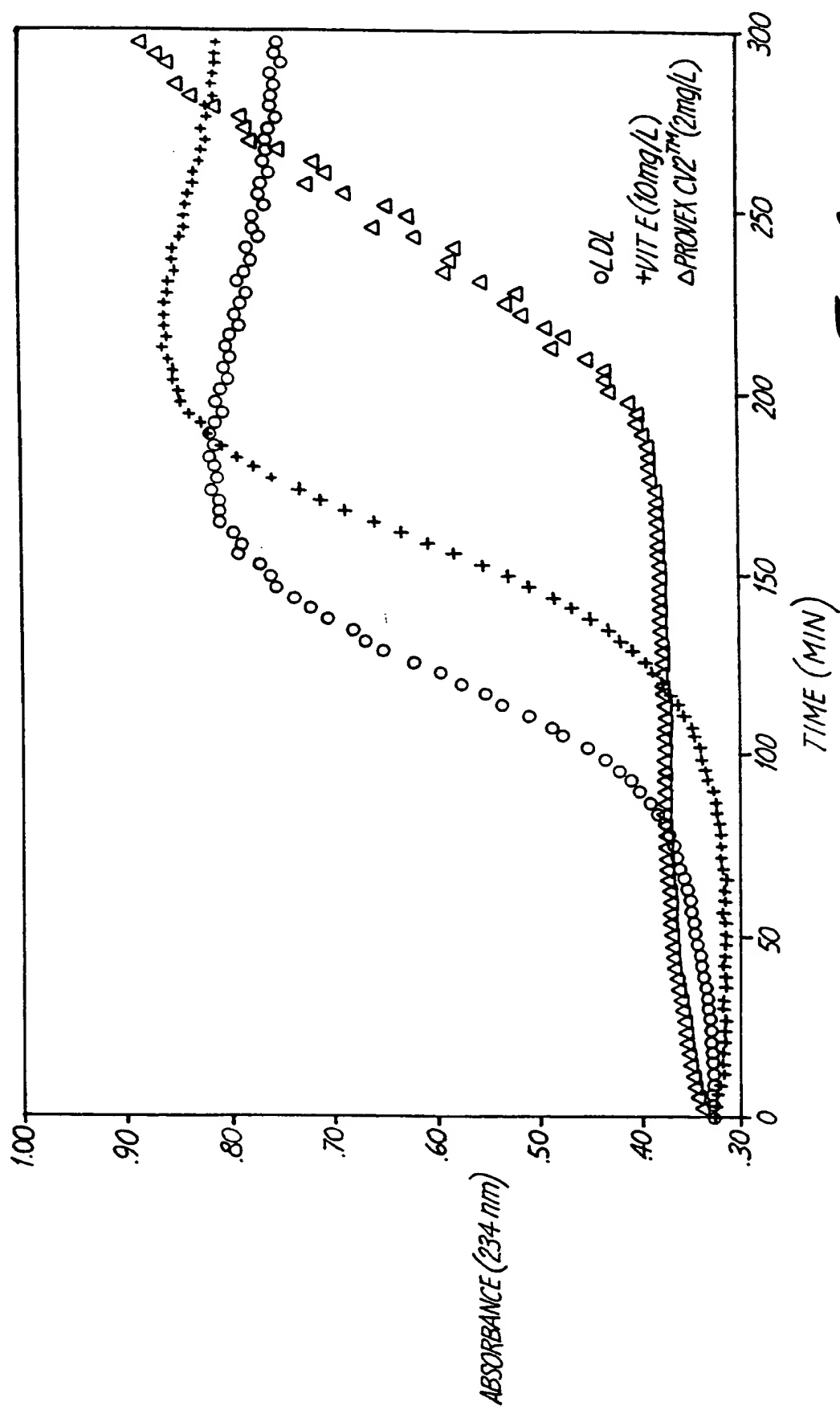


Fig. 8

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1651

PATENT COOPERATION TREATY

PCT

REC'D 27 APR 1999	
WIPO	PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT RECEIVED 18

(PCT Article 36 and Rule 70)

FEB 08 2000

Applicant's or agent's file reference 09143/005WO1		FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US98/16181	International filing date (day/month/year) 05 AUGUST 1998	Priority date (day/month/year) 06 AUGUST 1997	
International Patent Classification (IPC) or national classification and IPC IPC(6): A61K 35/78, 31/685, 31/715 and US Cl.: 424/94.66, 195.1			
Applicant MELALEUCA, INCORPORATED			

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 3 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 0 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand 02 MARCH 1999	Date of completion of this report 08 APRIL 1999
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer FREDERICK KRASS <i>gab for</i>
Facsimile No. (703) 305-3230	Telephone No. (703) 308-1235

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US98/16181

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments)*:

- ☒ the international application as originally filed.
- ☒ the description, pages 1-30 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____
pages _____ , filed with the letter of _____
- ☒ the claims, Nos. 1-37 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____
Nos. _____ , filed with the letter of _____
- ☒ the drawings, sheets/fig 1-8 , as originally filed.
sheets/fig NONE , filed with the demand.
sheets/fig NONE , filed with the letter of _____
sheets/fig _____ , filed with the letter of _____

2. The amendments have resulted in the cancellation of:

- ☒ the description, pages NONE .
- ☒ the claims, Nos. NONE .
- ☒ the drawings, sheets/fig NONE .

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US98/16181

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. STATEMENT**

Novelty (N)

Claims	<u>1-37</u>	YES
Claims	<u>NONE</u>	NO

Inventive Step (IS)

Claims	<u>1-37</u>	YES
Claims	<u>NONE</u>	NO

Industrial Applicability (IA)

Claims	<u>1-37</u>	YES
Claims	<u>NONE</u>	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-37 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest dietary supplements comprising at least one flavonoid source and an enzyme, said supplement being effective for inhibiting platelet activity and LDL cholesterol oxidation in a mammal at a dosage of about 30 mg/Kg or less.

Claims 1-37 meet the criteria set out in PCT Article 33(4); the applicability of the claimed nutritional supplements to the pharmaceutical/medical industries is self-evident.

----- NEW CITATIONS -----
NONE